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Abstract: AIMS The serotonin 2A receptor (HTR2A) is widely expressed in the brain and involved in the modulation of fear, mood, anxiety and other symptoms. HTR2A and HTR2A gene variations are implicated in depression, schizophrenia, anxiety and obsessive-compulsive disorder. To understand HTR2A signalling changes in psychiatric or neurodegenerative disorders, its normal pattern of brain expression and region specificity during development and aging needs to be clarified. The aim of the present study was to assess HTR2A expression through developmental and aging stages in six brain regions in postmortem human brain samples from individuals with no clinical or neuropathological evidence of neuropsychiatric disorders and to investigate the interaction with the rs6311 HTR2A promoter polymorphism. METHODS DNA, RNA and protein were isolated from postmortem brain samples including six regions (frontal cortex, striatum, amygdala, thalamus, brain stem and cerebellum) from 55 individuals. HTR2A mRNA levels were assessed using quantitative real time RT-PCR, and HTR2A protein levels - with western blot. The rs6311 HTR2A polymorphism was analyzed with genotyping. RESULTS We found that HTR2A mRNA and protein levels are differentially regulated with age in different brain regions studied, but are not affected by gender. Significant changes in HTR2A expression with age were found in frontal cortex, amygdala, thalamus, brain stem, and cerebellum. CONCLUSIONS Our results show plasticity and region specificity of HTR2A expression regulation in human brain with age, which may be important for the interaction with other neurotransmitter systems and for the occurrence of developmental periods with increased vulnerability to neuropsychiatric or neurodegenerative disorders.

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Abstract

Aims: The serotonin 2A receptor (HTR2A) is widely expressed in the brain and involved in the modulation of fear, mood, anxiety **and other symptoms**. HTR2A and *HTR2A* gene variations are implicated in depression, schizophrenia, anxiety and obsessive-compulsive disorder. To understand HTR2A signalling changes in psychiatric or neurodegenerative disorders, its normal pattern of brain expression and region specificity during development and aging needs to be clarified. The aim of the present study was to assess *HTR2A* expression through developmental and aging stages in six brain regions in postmortem human brain samples from individuals with no clinical or neuropathological evidence of neuropsychiatric disorders and to investigate the interaction with the rs6311 *HTR2A* promoter polymorphism. **Methods:** DNA, RNA and protein were isolated from postmortem brain samples including six regions (frontal cortex, striatum, amygdala, thalamus, brain stem and cerebellum) from 55 individuals. *HTR2A* mRNA levels were assessed using quantitative real time RT-PCR, and HTR2A protein levels – with western blot. The rs6311 *HTR2A* polymorphism was analyzed with genotyping. **Results:** We found that *HTR2A* mRNA and protein levels are differentially regulated with age in different brain regions studied, **but are not affected by gender**. Significant changes in HTR2A expression with age were found in frontal cortex, amygdala, thalamus, brain stem, and cerebellum. **Conclusions:** Our results show plasticity and region specificity of HTR2A expression regulation in human brain with age, which may be important for the interaction with other neurotransmitter systems and for the occurrence of developmental periods with increased vulnerability to neuropsychiatric or neurodegenerative disorders.

Keywords: serotonin 2A receptor, postmortem human brain, development, aging, single nucleotide polymorphism

Abbreviations: OCD, obsessive-compulsive disorder; 5-HT, serotonin; HTR2A, serotonin 2A receptor; yr, years; qRT-PCR, quantitative real time RT-PCR; PMI, postmortem interval; g.w., gestational weeks

Introduction

The process of brain maturation extends from the fetal period, after birth and through adolescence, into young adulthood [1]. Neurodevelopmental factors have been implicated in the pathophysiology of many psychiatric disorders such as schizophrenia, autism, obsessive-compulsive disorder (OCD) and attention deficit hyperactivity disorder [2]. Analyzing the normal pattern of expression of neurotransmitter receptors and other signalling molecules in the brain during development and aging may help better understand the pathological deviations in their expression occurring in psychiatric disorders.

Serotonin (5-HT) is a neurotransmitter that regulates mood, anxiety, cognition, and developmental processes such as neurogenesis and axon branching [3]. The serotonin 2A receptor (HTR2A) is a serotonin receptor subtype, which plays an important role in the modulation of anxiety and mood [4, 5]. Furthermore, the *HTR2A* gene has been implicated by genetic studies in neuropsychiatric disorders as schizophrenia, depression, anxiety, OCD and eating disorders [6, 7]. One of the genetic polymorphisms of the *HTR2A*, associated with psychiatric disorders, including OCD, is the promoter -1438A/G polymorphism (rs6311) [8-10]. *HTR2A* is expressed at the mRNA level in a number of different brain regions, and there is a great variability in the level of expression based on brain regions [11]. A recent study has determined the developmental regulation of *HTR2A* mRNA levels among other serotonin receptor subtypes in prefrontal cortex [12]. However, developmental regulation of expression may be different among diverse brain regions. Furthermore, it is important to determine also the developmental regulation of protein HTR2A expression, to account for the action of additional post-transcriptional regulatory mechanisms.

The aim of current study was to determine in postmortem human brain samples the regulation of the *HTR2A* expression on the mRNA and protein level through different stages of development and aging in six brain regions. Furthermore, we assessed the effect of the *HTR2A* rs6311 polymorphism on *HTR2A* expression levels.

Materials and Methods

Subjects

Human postmortem brain samples were obtained from the Department of Neuropathology, Institute of Pathology, University of Würzburg, Germany (member of the BrainNet Europe-BNEII) and the London Neurodegenerative Diseases Brain Bank, United Kingdom. For all analyzed samples written consent for tissue donation has been given either by the individuals or the next of kin. The study was approved by the Cantonal Ethic

Commission of Zürich (Ref. Nr. EK: KEK-ZH-Nr. 2013-0177). Samples were obtained from 55 individuals with age ranging from 16 weeks of gestation to 91 years (yr) of age. Only samples from individuals with no clinical or neuropathological evidence of neuropsychiatric disorders were included in the current study. Tissue from six brain regions was collected: frontal cortex (n=54), striatum (n=52), amygdala (n=47), thalamus (n=52), brain stem (n=49) and cerebellum (n=47). Samples were divided in 5 age groups: fetal, 0-2 yr, 21-40 yr, 41-60 yr, and over 60 yr. Demographic characteristics for the 5 age groups are presented in Table 1. The causes of death for the subjects included in the study are presented in Supplementary Table S1.

DNA, RNA and protein isolation

Frozen tissue samples with weight range of 27 – 34 mg were processed for simultaneous DNA, RNA and protein isolation with the Qiagen AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Hombrechtikon, Switzerland). Briefly, tissue samples were disrupted and homogenized in the provided buffer using the TissueLyser II (Qiagen). DNA was eluted after applying the lysate on an AllPrep DNA spin column. After adding ethanol, the sample was applied to an RNeasy spin column, where total RNA was eluted. Proteins in the sample were precipitated and pelleted by centrifugation. Since the provided buffer in the kit for dissolving proteins interferes with protein determination and did not allow complete dissolving of proteins, protein eluates were acetone precipitated and redissolved in urea buffer (7M urea, 2M thiourea, 2% CHAPS and trace bromophenol, pH 8).

Quantitative Real Time RT-PCR (qRT-PCR) Analysis

RNA concentrations, A260/A280 and A260/A230 ratios were measured with the NanoVue Plus spectrophotometer (GE Healthcare). **In a subset of samples RNA integrity was also analyzed with the Experion automated electrophoresis system (Bio-Rad, Reinach, Switzerland)**. 500 ng mRNA were reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad). qRT-PCR analysis was carried out using QuantiFast SYBR Green PCR kit (Qiagen) and primers specific for the human *HTR2A* gene (primer assay number QT00054306, Qiagen), and the following reference genes: *β-actin* (*ACTB)* (QT01680476), *aminolevulinate synthetase* (*ALAS1)* (QT00073122), *ribosomal protein L13a* (*RPL13A)* (QT00089915), *alanyl-tRNA synthetase* (*AARS)* (QT00054747), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH)* (QT01192646), *peptidyl prolyl isomerase A* (*PPIA)* (QT00866137), *ribosomal RNA* (*R18S)* (QT00019936), and *X-prolyl aminopeptidase 1* (*XPNPEP1)*

(QT00051471). **Eight reference genes were selected for testing in the current study based on previous investigations on reference genes stability in human postmortem tissue [13, 14].** Mean PCR efficiencies were in the range between 78 and 83 % for all the amplicons studied, and were calculated using the "LinRegPCR program" [15]. Quantification of normalized *HTR2A* mRNA levels was carried out using the "Biogazelle qBASE plus" software (Biogazelle). The "Biogazelle qBASE plus" program utilizes gene specific PCR efficiency correction, selects automatically reference genes, by taking into account their stability for normalization and carries out multiple reference gene normalization [16].

Genotyping for the rs6311 HTR2A polymorphism

DNA isolated from frontal cortex and in one case cerebellum was used for genotyping. DNA concentrations and A260/A280 and A260/A230 ratios were determined on the NanoVue Plus spectrophotometer. For genotyping the rs6311 *HTR2A* polymorphism, DNA, SNP genotyping assay for rs6311 (assay number C_8695278_10, Life Technologies) and TaqMan universal PCR master mix (Life Technologies) were combined in the 384-well plate. They were analyzed with the TaqMan SNP Genotyping Assay PCR standard protocol of Applied Biosystems, 07/2010 on a C1000 CFX384 thermal cycler (Bio-Rad). Analysis of the genotypes was performed with allelic discrimination program on the iCycler software (Bio-Rad). Bio-Rad CFX Manager software 2.1 was used for the analysis of the results. The allele frequencies did not deviate from the Hardy-Weinberg equilibrium, assessed with the Had2Know Hardy-Weinberg equilibrium calculator for two alleles (http://www.had2know.com/academics/hardy-weinberg-equilibrium-calculator-2-alleles.html).

Western blot analysis

Determination of protein levels was carried out with the Bradford assay (Sigma-Aldrich) [17]. Protein lysates were mixed with loading buffer and reducing agent (both from Life Technologies) and heated at 46 °C for 10 min. Lysates were then loaded on NuPage 4-12% Bis-Tris gels (Life Technologies) and after gel electrophoresis transferred onto nitrocellulose membranes with the iBlot western blotting system (Life Technologies). Membranes were blocked with 5% non-fat milk in phosphate buffered saline with 0.1% Tween 20 (PBST), and incubated with primary antibody against HTR2A (A-4, diluted 1:500 to 1:1000 in 5% nonfat dry milk-PBST for different brain regions, Santa Cruz Biotechnology) or β-actin (diluted 1:10000 in 5% nonfat dry milk-PBST, Santa Cruz Biotechnology)

overnight at 4 °C. Membranes were washed three times with PBST, and incubated with anti-mouse secondary HRP-conjugated antibody (diluted 1:2000 in 5% nonfat dry milk-PBST, Santa Cruz Biotechnology) for one hour at room temperature. Protein visualization was carried out with ECL Advance (GE Healthcare, Glattbrugg, Switzerland) on ChemiDoc XRS+ (Bio-Rad). Results were quantified with ImageJ 1.47 software (NIH).

Statistical Analysis

Statistical analysis was carried out using SPSS 21 from IBM. Normality of the distribution of mRNA and protein data was assessed with the Kolmogorov-Smirnov test with Liliefors significance correction. **Both normal and no normal distribution for *HTR2A* mRNA and protein levels was observed between different brain regions. To ascertain consistency between statistical evaluations throughout the study, differences in *HTR2A* mRNA levels, protein levels and mRNA/protein ratios between the different age groups were analyzed by the nonparametric Kruskal-Wallis test, followed by Mann-Whitney test pair-wise comparisons with p-value threshold adjusted based on the number of comparisons ($p<0.005$). The only exception was the *HTR2A* mRNA/protein ratio in the thalamus, where correlation of the ratio with the postmortem interval (PMI) was detected, and analysis of covariance (ANCOVA) with PMI as covariate after logarithmic transformation of the data was used for assessment.** Correlations between age and *HTR2A* mRNA and protein levels were assessed using Spearman's rank correlation. Correlations between age and *HTR2A* mRNA and protein levels for statistical significance determination were carried for all samples in a certain brain region as one group. Correlations are presented graphically (Figures 1-6) with best fit lines for the fetal and 0-2 yr samples as one group and the 21-40 yr, 41-60 yr and over 60 yr samples as a second group for easier visualization of the results on the age scale. To study the effect of rs6311 genotype (AA, AG and GG) on *HTR2A* mRNA and protein expression, ANCOVA with age as a covariate was used (in brain regions where *HTR2A* mRNA and protein levels were not normally distributed, logarithmic transformation of the data was carried out for the ANCOVA test, which resulted in lognormal distribution).

Results

In our data set gender had no significant effect on *HTR2A* mRNA or protein levels in any of the brain regions studied (Mann-Whitney test, $p > 0.05$) (data not shown). **The length of PMI did not differ significantly between the different age groups – Kruskal-Wallis**

test λ^2(4, N=55)=5.32, p=0.256. There was no significant correlation between the PMI and *HTR2A* mRNA levels, protein levels and **mRNA/protein ratios in the studied brain regions (Spearman's correlation, p>0.05), with the exception of the *HTR2A* mRNA/protein ratio in thalamus (Spearman's correlation, p<0.05) (data not shown).**

Regulation of HTR2A mRNA and protein expression in frontal cortex with age

HTR2A mRNA levels in frontal cortex were lowest in the fetal period **and higher** in the 0-2 yr, 21-40 yr, 41-60 yr, and over 60 yr groups - Kruskal-Wallis test λ^2(4, N=50)=22.518, p < 0.001. Pair-wise comparisons, using Mann-Whitney test were significant (p<0.005) for - fetal vs. 0-2 yr; fetal vs. 21-40 yr; fetal vs. 41-60 yr; and fetal vs. over 60 yr groups (Figure 1A). There was statistically significant positive correlation between age and *HTR2A* mRNA levels in frontal cortex - $r_s(50) = 0.326$, p = 0.021 (Figure 1B).

HTR2A protein levels in frontal cortex were lowest in the fetal period and increased throughout the older age groups with highest levels in the above 60 yr age group - Kruskal-Wallis test λ^2(4, N=49)=28.065, p < 0.001. Pair-wise comparisons with Mann-Whitney test were significant (p<0.005) for - fetal vs. 21-40 yr; fetal vs. 41-60 yr; fetal vs. over 60 yr; and 0-2 yr vs. over 60 yr groups (Figure 1C). There was statistically significant positive correlation between age and HTR2A protein levels in frontal cortex - $r_s(49) = 0.737$, p<0.001 (Figure 1D).

Regulation of HTR2A mRNA and protein expression in striatum with age

Differences in *HTR2A* mRNA levels in striatum did not reach significance between the different age groups - Kruskal-Wallis test λ^2(4, N=47)=8.775, p=0.067 (Figure 2A). However, there was a statistically significant negative correlation between age and *HTR2A* mRNA levels in striatum: $r_s(47) = -0.466$, p = 0.001 (Figure 2B).

HTR2A protein levels in striatum also did not show significant differences between the age groups - Kruskal-Wallis test λ^2(4, N=37)=5.963, *p*=0.202 (Figure 2C). There was also no significant correlation between age and HTR2A protein levels in the striatum: $r_s(37) = 0.299$, p = 0.072 (Figure 2D).

Regulation of HTR2A mRNA and protein expression in amygdala with age

In amygdala *HTR2A* mRNA levels showed an increase in the older age groups - Kruskal-Wallis test λ^2(4, N=46)=10.421, p=0.034. Pair-wise comparisons, using Mann-Whitney test were significant (p<0.005) for - fetal vs. 41-60 yr; and fetal vs. over 60 yr

groups (Figure 3A). There was no significant correlation between age and *HTR2A* mRNA levels in the amygdala: $r_s(46) = 0.271$, $p = 0.068$ (Figure 3B).

HTR2A protein levels in amygdala were lowest in the fetal group and higher in the older age groups - **Kruskal-Wallis test $\lambda^2(4, N=40)=15.868$, $p=0.003$. Pair-wise comparisons, using Mann-Whitney test were significant ($p<0.005$) for** - fetal vs. 0-2 yr; fetal vs. 21-40 yr; fetal vs. 41-60 yr; and fetal vs. over 60 yr groups (Figure 3C). There was a positive correlation between age and HTR2A protein levels in amygdala: $r_s(40) = 0.557$, $p<0.01$ (Figure 3D).

Regulation of HTR2A mRNA and protein expression in thalamus with age

In thalamus there were no significant differences between the different age groups in *HTR2A* mRNA levels - **Kruskal-Wallis test $\lambda^2(4, N=46)=1.24$, $p=0.871$** (Figure 4A). There was also no statistically significant correlation between age and the *HTR2A* mRNA levels in the thalamus ($r_s(46) = -0.014$, $p >0.05$) (Figure 4B).

HTR2A protein levels in the thalamus were lowest in the fetal period, and increased in the 21-40 yr, 41-60 yr and over 60 yr age groups - **Kruskal-Wallis test $\lambda^2(4, N=44)=26.974$, $p<0.001$. Pair-wise comparisons, using Mann-Whitney test were significant ($p<0.005$) for - fetal vs. 0-2 yr; fetal vs. 21-40 yr; fetal vs. 41-60 yr; fetal vs. over 60 yr; and 0-2 yr vs. over 60 yr groups** (Figure 4C). There was a positive correlation between age and HTR2A protein levels in the thalamus. The correlation between age and HTR2A protein levels was $r_s(44) = 0.738$, $p < 0.01$ (Figure 4D).

Regulation of HTR2A mRNA and protein expression in brain stem with age

HTR2A mRNA levels in the brain stem were higher in the fetal group compared to the over 60 yr group - Kruskal-Wallis test $\lambda^2(4, N=42)=12.872$, $p=0.012$. Pair-wise Mann-Whitney comparisons were significant for fetal vs. over 60 yr $p=0.002$ (Figure 5A). There was a negative correlation between age and *HTR2A* mRNA levels in the brain stem: $r_s(42) = -0.569$, $p < 0.01$ (Figure 5B).

HTR2A protein levels in the brain stem were lowest in the fetal period and increased in the older age groups - **Kruskal-Wallis test $\lambda^2(4, N=42)=13.249$, $p=0.01$. Pair-wise Mann-Whitney comparisons were significant for:** fetal vs. 0-2 yr; fetal vs. 21-40 yr; fetal vs. 41-60 yr; and fetal vs. over 60 yr (Figure 5C). There was a positive correlation between age and HTR2A protein levels in the brain stem: $r_s(42) = 0.426$, $p < 0.01$ (Figure 5D).

Regulation of HTR2A mRNA and protein expression in cerebellum with age

HTR2A mRNA levels in the cerebellum decreased with age with highest levels in the fetal period and 0-2 yr groups and lowest in the 41-60 yr and over 60 yr age groups - Kruskal-Wallis test $\lambda^2(4, N=43)=19.056$, $p=0.001$; pair-wise Mann-Whitney comparisons were significant ($p<0.005$) for fetal vs. 41-60 yr; fetal vs. over 60 yr; 0-2yr vs. 41-60 yr; and 0-2 yr vs over 60 yr groups (Figure 6A). There was a negative correlation between age and *HTR2A* mRNA levels in cerebellum: $r_s(43) = -0.53$, $p < 0.01$ (Figure 6B).

HTR2A protein levels in the cerebellum increased with age with lowest levels in the fetal and 0-2 yr groups and highest in the 41-60 yr and over 60 yr groups - Kruskal-Wallis test $\lambda^2(4, N=40)=22.748$, $p=0.001$; pair-wise Mann-Whitney comparisons were significant ($p<0.005$) for: fetal vs. 41-60 yr; fetal vs. over 60 yr; 0-2 yr vs. 41-60 yr; and 0-2 yr vs over 60 yr groups (Figure 6C). There was a positive correlation between age and HTR2A protein levels in cerebellum: $r_s(40) = 0.54$, $p < 0.01$ (Figure 6D).

Alterations in HTR2A mRNA/protein ratios in different brain regions with age

The *HTR2A* mRNA/protein ratios in the different age groups were studied. *HTR2A* mRNA/protein ratios were negatively correlated with age in all brain regions (Supplementary Table S2) - r_s = -0.629 for frontal cortex, -0.491 for striatum, -0.33 for amygdala, -0.606 for thalamus, -0.607 for brain stem, -0.588 for cerebellum, $p<0.05$ for all regions. There was a consistent trend for highest *HTR2A* mRNA/protein ratios in the fetal group and lower *HTR2A* mRNA/protein ratios in the older age groups throughout all brain regions studied, even though its extent showed variation between the brain regions (Supplementary Figure S1). The differences were significant in frontal cortex ($\lambda^2(4, N=46)=19.527$, $p=0.001$), brain stem ($\lambda^2(4, N=36)=14.465$, $p=0.006$), and cerebellum ($\lambda^2(4, N=38)=24.906$, $p<0.001$) assessed with Kruskal-Wallis test. They were also significant in thalamus assessed with ANCOVA with PMI as covariate, followed by LSD post-hoc test ($p<0.05$). The differences did not reach significance for striatum ($\lambda^2(4, N=33)=7.446$, $p=0.114$) and amygdala ($\lambda^2(4, N=40)=6.393$, $p=0.172$) assessed with Kruskal-Wallis test.

rs6311 HTR2A polymorphism and HTR2A mRNA and protein expression

No significant differences in *HTR2A* mRNA or protein levels were detected among the rs6311 *HTR2A* genotypes (AA, AG, GG) in any of the brain regions studied (ANCOVA with age as covariate, $p>0.05$) (Figure 7A-L).

Discussion

In the current study *HTR2A* mRNA and protein expression levels during different stages of development and aging and in six brain regions were analyzed using postmortem human brain samples from individuals with no clinical or neuropathological evidence of neuropsychiatric disorders. Our data showed regulation of HTR2A expression with age that was specific for each brain region studied. In particular, significant differences in HTR2A expression between different age groups were found in the frontal cortex (mRNA and protein levels), amygdala (**mRNA and** protein levels), thalamus (protein levels), brain stem (mRNA and protein levels), and cerebellum (mRNA and protein levels). Furthermore, the fetal period was identified as the age group, which differed most strongly in its HTR2A expression in comparison to all other age groups studied.

The HTR2A receptor has been implicated by postmortem, genetic and imaging studies in a number of psychiatric disorders [6]. Thus, changes in HTR2A expression levels in postmortem brain have been detected in schizophrenia [18-20]. In line with these findings, a recent meta-analysis confirmed a significant association of the rs6311 *HTR2A* polymorphism with schizophrenia [21]. Previous postmortem studies have observed changed HTR2A levels in brains from individuals with major depression **(in prefrontal cortex and hippocampus)** [4, 22], and suicide victims **(in frontopolar and prefrontal cortex, hippocampus and amygdala)** [23-26].

Animal studies have suggested that windows of vulnerability **to environmental influences** may exist in neuronal development, disruptions in signaling during which may be particularly damaging and increasing the vulnerability to psychiatric disorders [27]. In order to understand changes occurring in psychiatric disorders, it is important to analyze the normal developmental pattern of signaling molecules in the brain [28]. In our study HTR2A expression levels showed greatest differences compared to other age groups in the fetal group, underscoring this developmental period as particularly important for the plastic regulation of expression. Our data are in agreement with a recent transcriptome and genome study of prefrontal human cortex, which identified fetal development as the period during which overall gene expression changes occur much faster compared to other age periods [29].

Interestingly, in the oldest age group in the current study – over 60 yr, HTR2A protein levels were generally relatively higher compared to the mRNA levels for the corresponding brain region. This effect could be specific to the HTR2A receptor or a more widely occurring

phenomenon. It could be related to alterations in post-transcriptional modifications with age – for example related to the ubiquitin-proteasome system or microRNA regulation and warrants further investigation [30, 31].

Our study involved six brain regions – frontal cortex, striatum, amygdala, thalamus, brain stem and cerebellum. We found that regulation of HTR2A expression with age was specific for each brain region, emphasizing the importance of analyzing data from postmortem studies in the context of the brain region studied. These regional specificities in regulation are important in light of the potential for interaction with different neurotransmitter systems and the function of the HTR2A receptor in each brain area. **While the process of brain development is very complex and encompasses a long period of time, some regional specificities can be noted. They can be related to the processes of neuronal plasticity and activity, as well as to synaptic density. For example, the processes of synaptogenesis and synapse elimination, which are an essential part of brain development, apparently occur with varying time courses in different regions of the human cerebral cortex [32, 33].**

The levels of serotonin and its metabolites have been studied in postmortem human brain. In putamen, increase in serotonin levels and decrease in the levels of its metabolite 5-hydroxyidoleacetic acid have been observed with age, starting from the second decade of life [34]. The dependence of monoamine oxidase A activity, which is particularly important for serotonin metabolism, on age in frontal cortex has also been investigated. A fast decrease in monoamine oxidase A activity in postmortem frontal cortex in the first two years after birth and stable levels afterwards has been observed [35]. A direct correlation between serotonin levels alterations with age from previous studies and our results on HTR2A levels is difficult due to the diverse brain regions and age ranges studied.

The rs6311 *HTR2A* polymorphism has been associated with potential changes in *HTR2A* expression levels [36], but these findings have not been consistently replicated [37-39]. A recent study also suggested that the rs6311 polymorphism decreases the usage of an upstream transcription start site, which encodes a longer 5'-untranslated region with higher translation efficiency [7]. We assessed the effect of the rs6311 polymorphism on the *HTR2A* mRNA and protein levels in the different brain regions studied and did not find any statistically significant differences between the genotypes. The observed lack of the rs6311 *HTR2A* polymorphism on HTR2A expression levels may be related to the relatively small sample size in our study. In addition, *HTR2A* mRNA and protein expression was correlated

with age in a number of brain regions in our study. An investigation including a larger number of samples closely matched by age is warranted to conclusively assess the effect of the rs6311 *HTR2A* polymorphism of *HTR2A* expression levels in postmortem brain samples.

In conclusion, our results showed regulation of *HTR2A* mRNA and protein levels in postmortem brain with age, which was brain region-specific. The data of the current study will contribute to better understanding of the regulation of *HTR2A* brain expression in development and aging.

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Authors contributions:

E.G., C.M.M. and Z.M. designed the experiments; Z.M. and S.F. performed experiments and analyzed data; Z.M. wrote the manuscript; E.G., C.M.M. and S.W. revised the manuscript; S.W. provided financial support for the study.

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Figures legends:

Figure 1. Developmental regulation of *HTR2A* mRNA and protein expression in frontal cortex. **A.** *HTR2A* mRNA levels in frontal cortex across the different age groups presented as mean±SEM. *HTR2A* mRNA levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests ($p<0.005$). **B.** Correlation between age and *HTR2A* mRNA levels in frontal cortex - $r_s(50) = 0.326$, $p = 0.021$, linear fit lines and 95% confidence interval (CI) included. **C.** HTR2A protein levels in frontal cortex across the different age groups presented as mean±SEM. HTR2A protein levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests ($p<0.005$). **D.** Correlation between age and HTR2A protein levels in frontal cortex - $r_s(49) = 0.737$, $p < 0.001$, linear fit lines and 95% CI included.

Figure 2. Developmental regulation of *HTR2A* mRNA and protein levels in striatum. **A.** *HTR2A* mRNA levels in striatum across the different age groups presented as mean±SEM. **B.** Correlation between age and *HTR2A* mRNA levels in striatum - $r_s(47) = -0.466$, $p = 0.001$, linear fit lines and 95% CI included. **C.** HTR2A protein levels in striatum across the different age groups presented as mean±SEM. **D.** Correlation between age and HTR2A protein levels in striatum, linear fit lines and 95% CI included.

Figure 3. Developmental regulation of HTR2A mRNA and protein levels in amygdala. **A.** *HTR2A* mRNA levels in amygdala across the different age groups presented as mean±SEM. ***HTR2A* mRNA levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests ($p<0.005$)**. **B.** Correlation between age and *HTR2A* mRNA levels in amygdala, linear fit lines and 95% included. **C.** HTR2A protein levels in amygdala across the different age groups presented as mean±SEM. HTR2A protein levels change significantly through development **assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests ($p<0.005$)**. **D.** Correlation between age and HTR2A protein levels in amygdala - $r_s(40) = 0.557$, $p<0.01$, linear fit lines and 95% CI included.

Figure 4. Developmental regulation of *HTR2A* mRNA and protein levels in thalamus. **A.** *HTR2A* mRNA levels in thalamus across the different age groups presented as mean±SEM. **B.** Correlation between age and *HTR2A* mRNA levels in thalamus, linear fit lines and 95% CI included. **C.** HTR2A protein levels in thalamus across the different age groups presented as mean±SEM. HTR2A protein levels change significantly through development **assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests (p<0.005). D.** Correlation between age and HTR2A protein levels in thalamus - $r_s(44) = 0.738$, $p < 0.01$, linear fit lines and 95% CI included.

Figure 5. Developmental regulation of *HTR2A* mRNA and protein levels in brain stem. **A.** *HTR2A* mRNA levels in brain stem across the different age groups presented as mean±SEM. *HTR2A* mRNA levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests (p<0.005). **B.** Correlation between age and *HTR2A* mRNA levels in brain stem - $r_s(42) = -0.569$, $p < 0.01$, linear fit lines and 95% CI included. **C.** HTR2A protein levels in brain stem across the different age groups presented as mean±SEM. HTR2A protein levels change significantly through development **assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests (p<0.005). D.** Correlation between age and HTR2A protein levels in brain stem - $r_s(42) = 0.426$, $p < 0.01$, linear fit lines and 95% CI included.

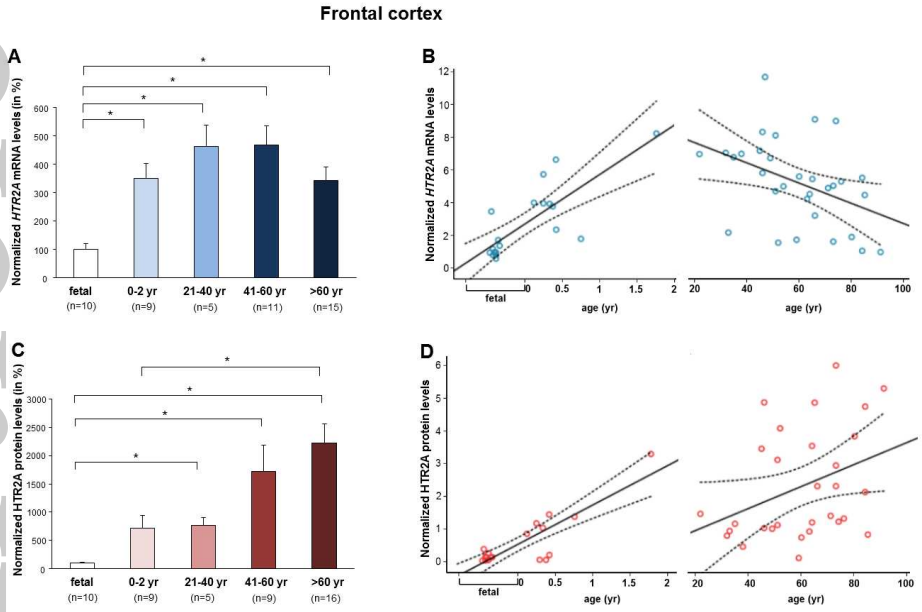
Figure 6. Developmental regulation of *HTR2A* mRNA and protein levels in cerebellum. **A.** *HTR2A* mRNA levels in cerebellum across the different age groups presented as mean±SEM. *HTR2A* mRNA levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests (p<0.005). **B.** Correlation between age and *HTR2A* mRNA levels in cerebellum $r_s(43) = -0.53$, $p < 0.01$, linear fit lines and 95% CI included. **C.** HTR2A protein levels in cerebellum across the different age groups presented as mean±SEM. HTR2A protein levels change significantly through development assessed with Kruskal-Wallis test, * presents statistical significance between groups assessed with Mann-Whitney tests (p<0.005). **D.** Correlation between age and HTR2A protein levels in cerebellum - $r_s(40) = 0.54$, $p < 0.01$, linear fit lines and 95% CI included.

Figure 7. *HTR2A* mRNA and protein levels in different brain regions of subjects grouped depending on their rs6311 *HTR2A* genotype (AA, AG, GG). *HTR2A* mRNA levels in frontal cortex (**A**), striatum (**B**), amygdala (**C**), thalamus (**D**), brain stem (**E**) and cerebellum (**F**) between different rs6311 *HTR2A* genotypes. HTR2A protein levels in frontal cortex (**G**), striatum (**H**), amygdala (**I**), thalamus (**J**), brain stem (**K**) and cerebellum (**L**) between different rs6311 *HTR2A* genotypes. Both *HTR2A* mRNA and protein levels are presented as mean±SEM.

Table 1. Demographic characteristics of the subjects included in the study by age group.

Age groups	Gender	Average age ± SD	PMI ± SD (h)
fetal	7 F / 4 M	21.3 ± 6.86 g. w.	36.9 ± 19.9
0-2 yr	5 F / 6 M	0.62 ± 0.6 yr	25.5 ± 7.7
21-40 yr	2 F / 3 M	32 ± 6 yr	25.8 ± 18.3
41- 60 yr	5 F / 6 M	50.9 ± 5.1 yr	26.9 ± 12.5
over 60 yr	6 F / 11 M	73.6 ± 8.6 yr	25.9 ± 10.2

Abbreviations: F=female, M=male, yr=years, g. w.=gestational weeks, PMI=postmortem interval, h=hours



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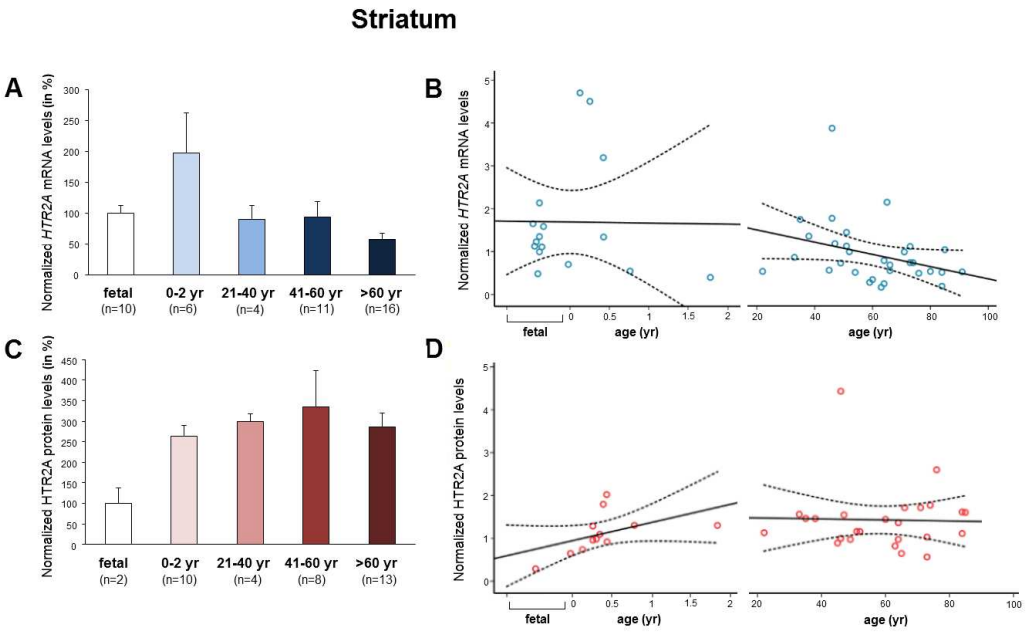

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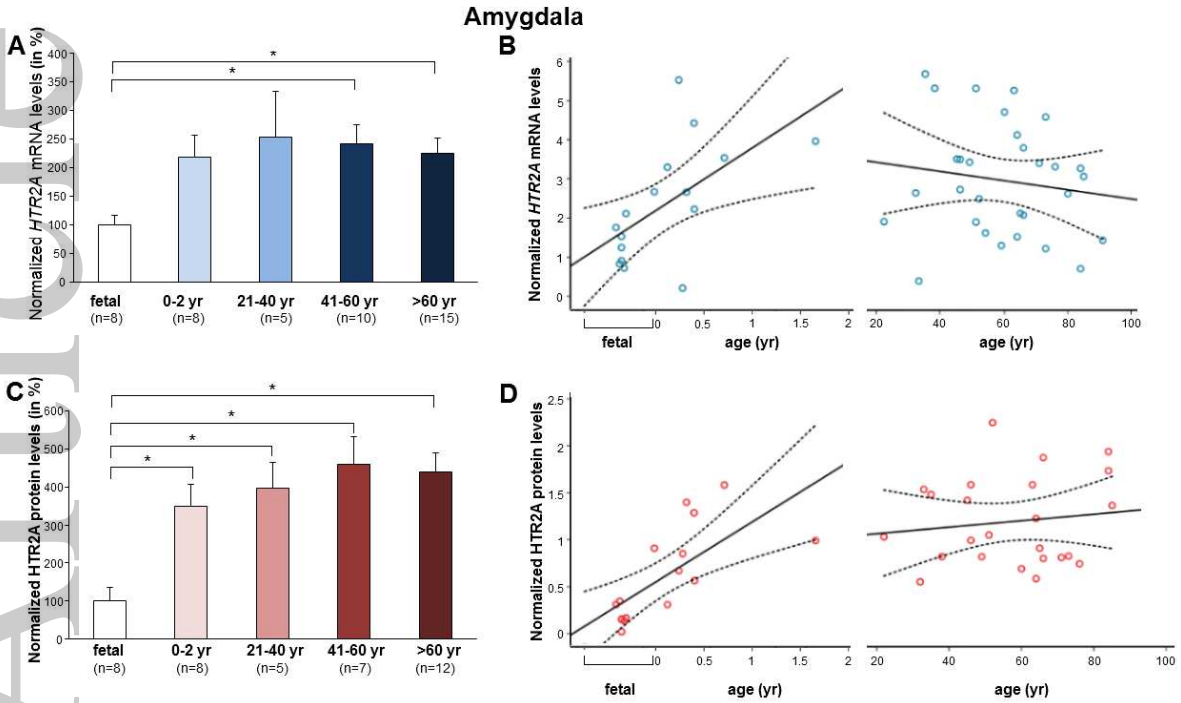

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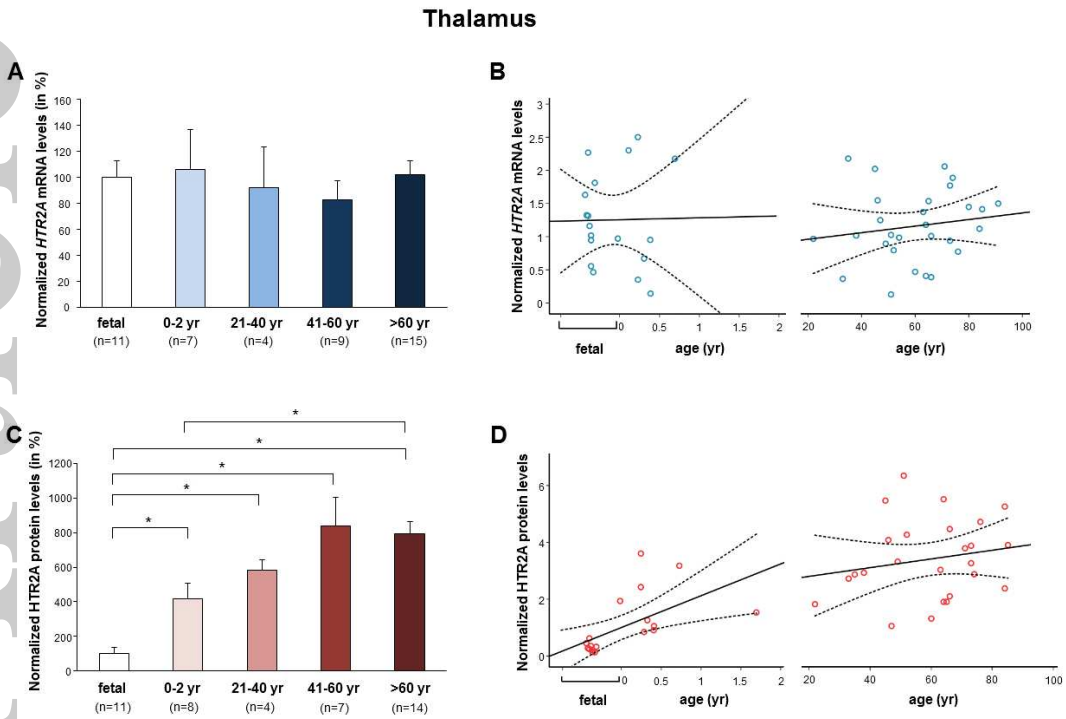

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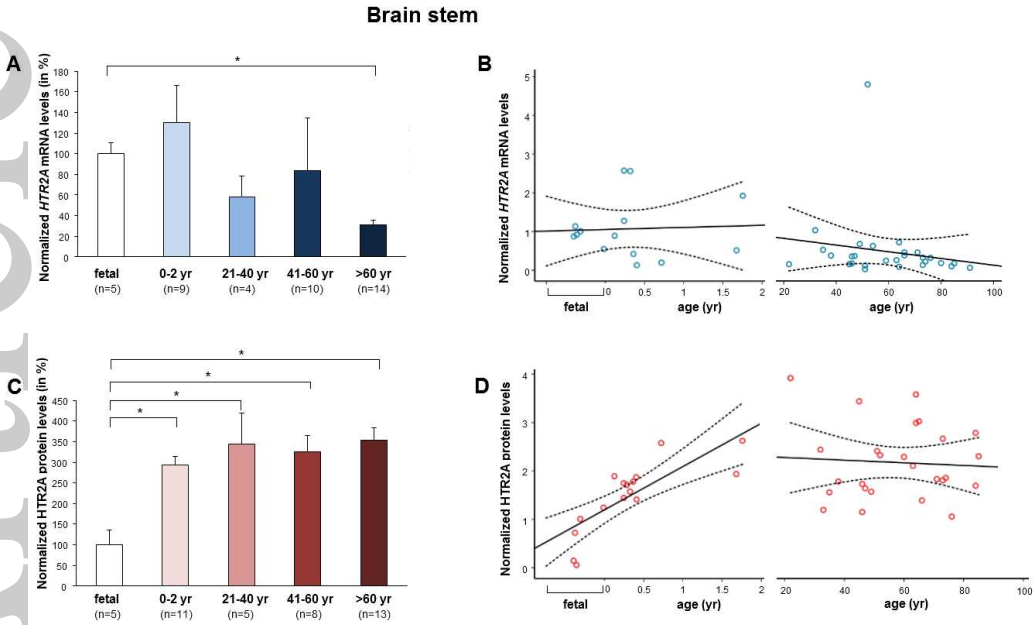

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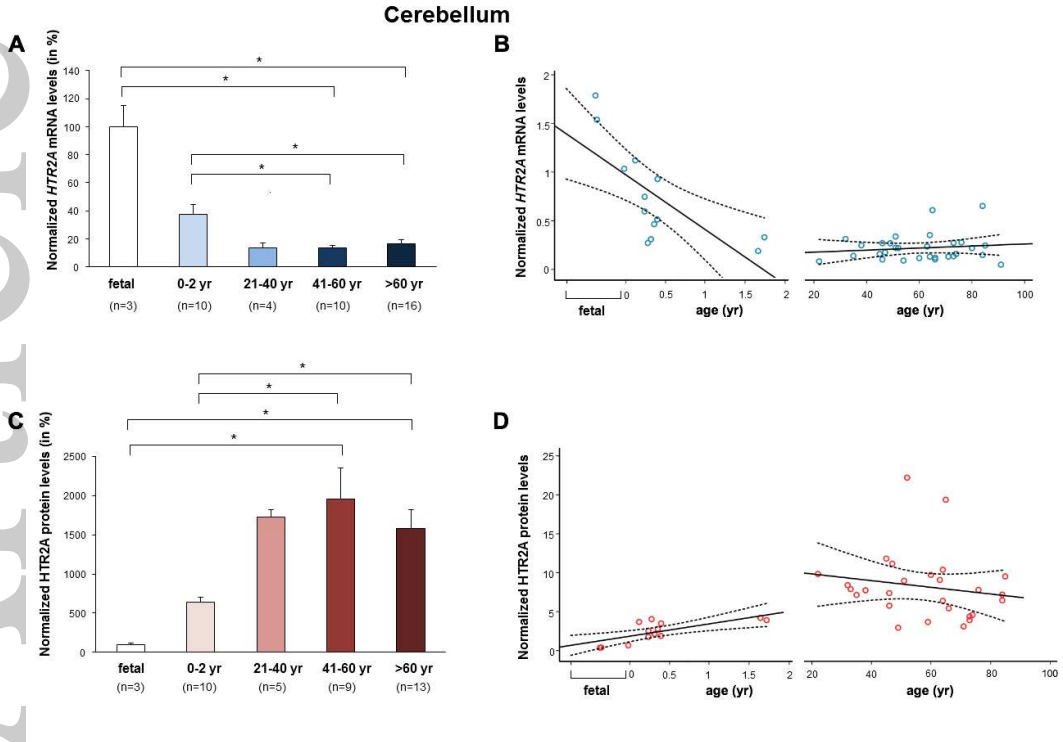

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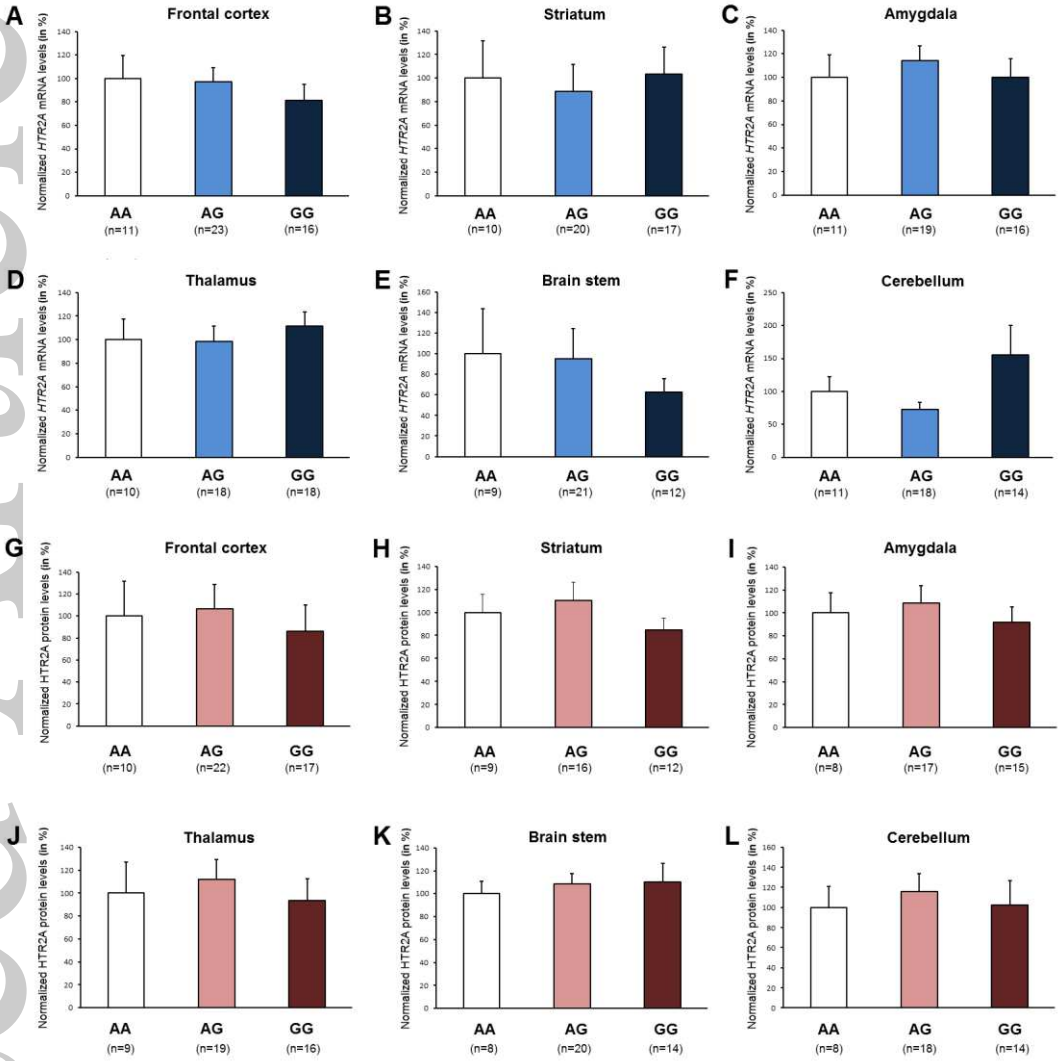

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